Quantifying Polarized Extracellular Matrix Secretion in Cultured Endothelial Cells

Chapter 17

Quantifying polarized extracellular matrix secretion in cultured endothelial cells

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Abstract

In endothelial cells (ECs), the onset of apico-basal polarity is primarily regulated by the interaction of integrins with the surrounding extracellular matrix (ECM). ECs secrete and polymerize fibronectin (FN), a unique, permissive substrate that allows vascular morphogenesis and lumen formation. We previously identified a signaling pathway that, under the control of the adhesion site adaptor protein PPFIA1, integrates the polarized secretion of freshly synthesized FN with the recycling of conformationally active α5β1 integrin, the main FN receptor in ECs. To characterize the functional role of PPFIA1-dependent signaling in ECs, we set up a Transwell-based assay to quantify the polarized secretion of ECM proteins. To this aim, we allowed ECs to form a confluent monolayer on

the Transwell membrane and checked its integrity by measuring transendothelial electric resistance and controlling the stability of tight junctions over time by fluorescent confocal microscope analysis. Finally, we quantified apical and basolateral FN secretion in control and PPFIA1-silenced EC culture medium by western blot analysis coupled to spike-in normalization.

Key Words: Fibronectin, Integrins, Cell polarity, Secretion, tight junctions, Golgi

Running title: Polarized endothelial ECM secretion

1 Introduction

Endothelium, which constitutes the inner cellular lining of all blood vessels, is formed by flat yet highly polarized cells, with the apical surface facing the lumen and the basolateral side contacting the basement membrane. During vascular morphogenesis, the onset of endothelial cell (EC) apico-basal polarity is initiated by the interaction of integrins with the surrounding extracellular matrix (ECM) [1]. Fibronectin (FN) and type I collagen play a permissive role during blood vessel formation and the interaction with their receptors, the β1 family of integrins (α2β1, α1β1, α5β1, and α4β1), and αv containing dimers αvβ3 and αvβ5, induces lumen formation [2]. Once established, EC apico-basal polarity is sustained by the unique sorting properties of the trans-Golgi network (TGN) and reinforced by the continuous turnover of the ECM components and their receptors at adhesion sites [3].

ECs produce and secrete FN through the basolateral pole, giving rise to an abluminal fibrillar meshwork that also incorporates other ECM proteins. Unlike exogenous soluble plasma FN, endogenous cellular FN is crucial to allow the formation of polymeric FN fibrils, the establishment of EC cell polarity, and the angiogenic remodeling of blood vessels [4,5]. Therefore, it is critical to develop quantitative assays aimed at analyzing polarized FN secretion by an intact EC monolayer. Recently, we discovered a signaling pathway that, under the control of the adhesion site adaptor protein PPFIA1, couples polarized FN secretion and conformationally active α5β1 integrin recycling and supports vascular morphogenesis both *in vitro* and in the developing zebrafish embryo [4].

To analyze polarized FN secretion in control or PPFIA1 silenced ECs, we set up a Transwell based secretion assay. To this aim, we seeded human arterial endothelial cells (AECs), extracted from blood cords, on Transwell insert filters with pore size of 0.4 micron, which prevents EC migration through them. When AECs form a confluent monolayer, the upper compartment of the Transwell insert is completely separated from the lower one, and the polarized apical and basolateral secretion of soluble proteins can be evaluated. To monitor the integrity of the endothelial monolayer, we measured the trans-endothelial electrical resistance (TEER) by applying an electrical signal across electrodes placed on both sides of the Transwell insert membrane and measuring the resulting current flow. To further assess the stability of the endothelial monolayer, we analyzed by fluorescent

confocal microscopy EC tight junctions, that is, the intercellular adhesive connections that seal intercellular clefts, thus allowing the development of a selectively permeable endothelial interface [6]. We fixed AECs on Transwell polycarbonate membrane (suitable for optical imaging), and stained them with anti-vascular endothelial (VE)-cadherin and anti-zonula occludens 1 (ZO1) antibodies. Immunofluorescence analyses revealed that, 24 hours after seeding, AECs form a confluent monolayer, which is maintained until the end of the assay, 72 hours later.

We monitored AEC FN secretion over time and noticed that, 96 hours after cell seeding, endogenous FN accumulates in the culture medium. Even if mainly secreted basolaterally, it was possible to evaluate the difference between the FN amounts released by control or PPFIA1 silenced ECs in both upper and lower chambers. FN exists in multiple isoforms, among which plasma FN, a soluble form produced by hepatocytes and present in serum, and cellular FN, which is produced by different cell types, *e.g.* ECs or vascular smooth muscle cells or myofibroblasts, during active tissue morphogenesis [7,8]. Differently from its plasmatic isoform, cellular FN can specifically contain FN type III repeat harboring extra domains A and B (EDA and EDB) that are encoded by alternatively spliced exons [9]. Hence, to avoid cross-contamination by any trace of serum FN and to selectively monitor the secretion of cellular FN by AEC in the culture medium, we decided to use IST9 mouse monoclonal antibody (Mab), which is specifically directed against the EDA domain of human cellular FN [10].

2 Materials

1. Human umbilical arterial endothelial cells (AECs): cells were extracted from the artery of human umbilical cords, as previously described [4].
2. EGM-2 Endothelial Cell Growth Medium-2 (Lonza).
3. Empty Columns PD-10 (GE Healthcare).
4. Gelatin Sepharose 4B (GE Healthcare).
5. Phosphate Buffered Saline (PBS) 1X: Dulbecco's PBS.
6. Trypsin-EDTA Solution 1X.
7. 1% gelatin solution, gelatin powder from porcine skin (Sigma Aldrich).
8. siGENOME Control Non-Targeting siRNA #1 (Thermo Scientific, Dharmacon).
9. siGENOME SMART pool Human PPFIA1 (Thermo Scientific, Dharmacon).
10. Oligofectamine Reagent (Invitrogen).
11. Opti-MEM I Reduced Serum Medium (Life Technologies).
12. Countess Automated Cell Counter (Life Technologies).
13. Polycarbonate membrane Transwell inserts, pore 0.4μm (Corning).
14. Multiple Well cell culture Plates, 24-wells and 6-wells.
15. Millicell ERS-2 Epithelial Volt-Ohm Meter (Millipore).
16. 70% ethanol solution.
17. Small screwdriver for the R. Adj. screw of the ERS-2 Epithelial Volt-Ohm Meter.
18. Rabbit anti-Mouse IgG, IgM (H+L) Secondary Antibody (Invitrogen).
19. SDS–Page Protein Sample Buffer (4X).
20. Polyacrylamide gel 8% for SDS-Page (Blot™ Bis-Tris Plus Gels, 10-well, Life technologies), nitrocellulose membrane (Bio-Rad, Hercules, CA, USA) and Trans-Blot® Turbo™ Transfer System (Bio-Rad, Hercules, CA, USA).
21. Clarity western ECL substrate (Bio-Rad).
22. 10% BSA in TBS, 0.1% Tween-20.
23. TBS 0.1% Tween-20.

24. Mouse Mab anti- ED-A Fibronectin (ED-A FN) IST9 (Santa Cruz Biotechnology).
25. Goat anti-Rabbit (Santa Cruz Biotechnology) and Goat anti-Mouse (Jackson ImmunoResearch Laboratories) HRP conjugated secondary antibodies.
26. Paraformaldehyde (PAF) solution 4% in PBS (ChemCruz).
27. Permeabilizing solution PBS 0.1% Triton X-100.
28. Primary and secondary blocking: PBS 1% Donkey Serum (Sigma Aldrich).
29. VE-cadherin Goat Polyclonal Antibody (Santa Cruz Biotechnology), ZO-1 Rabbit Polyclonal Antibody (Invitrogen).
30. Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 555 conjugated (Invitrogen) and Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 conjugated (Invitrogen).
31. DAPI (Life technologies), TO-PRO-3 (Life technologies).
32. Precision cover glasses thickness 12mm, No. 1.5H, (tol. ± 5 μm) (Marienfeld).
33. Superfrost Microscope Slides, 76 x 26 mm. Fluoromount-G (Invitrogen) or Mowiol 4-88 mounting medium, (*see* **Note 1)**.

3 Methods

3.1 siRNA-mediated gene silencing in AECs

1. The day before siRNA oligofection, seed 120,000 AECs/well (*see* **Note 2**) in 6-well plate, pre-coated with 1 ml/well of 1% gelatin solution for 5 minutes at 37°C, and culture in 1.5 ml/well EGM-2 medium.
2. Next day, wash cells twice with PBS, add 0.8 ml/well of OptiMEM-1 and place the cells back in the incubator at 37°C.
3. Prepare siRNA reaction for each experimental point: for each well prepare solution A, adding 4 µl of Oligofectamine to 11 µl of OptiMEM. Incubate 5' at room temperature (RT). Prepare Solution B, adding 10 µl of siRNA (200 pmol) to 175 µl of OptiMEM.
4. Mix solutions A and B, incubate the resulting mixture for 20' at RT. Add the mixture on cells, and put the plate back in the incubator for 4-6 hours at 37°C.
5. Replace the transfection medium with 1.5 ml/well EGM-2 medium.
6. Nextday, repeat the siRNA oligofection from step 2 to 5.
7. 24 hours after the second oligofection, AECs are ready for seeding on Transwell inserts (*see* **Note 2**).

3.2 Preparing FN-free EGM-2 medium

1. Prepare the empty column inserting the support for the Sepharose on the bottom of the column and equilibrate it with 5 ml of PBS.
2. Load a column with 1ml of slurry gelatin-Sepharose solution and let the resin settle on the bottom of the column. When all the resin is packed, wash it three times with 5 ml of PBS.
3. Load 10 ml of fetal bovine serum (the one provided in EGM-2 BulletKit or another one tested for endothelial cells - see **Note 2**) on the top of the column and let it slowly flow through the resin.
4. Collect the serum and reload it on the top of the column three times
5. Collect the serum and freeze it in small aliquots at -80°C.

6. Add all aliquots of supplements provided in the BulletKit to the EBM medium except for the serum. Collect a volume of medium suitable for the size of the experiment (sufficient for seeding the cells on Transwell chambers and replace the medium once, *see* Section 3.3) and add 2% of FN-free serum.

3.3 Seeding AECs on Transwell chambers

1. Coat the upper face of Transwell insert membrane adding 50 µl of 1% gelatin solution and incubate them hanging in a 24-well plate for 1 hour at 37°C.
2. In the meantime, remove the culture medium from the cells and wash them twice with PBS.
3. Incubate each well with 300 µl of trypsin-EDTA solution for 1 min at 37 °C to detach the cells and inactivate trypsin with 700 µl of EGM-2.
4. Collect the cell suspension and count them (we use the Countess system, from Thermo Fisher).
5. Centrifuge the cell suspension for 5 minutes at 800 rpm.
6. Suspend the pellet of AECs in FN-free EGM-2 to a final concentration of 150.000 cells/200 µl.
7. Carefully remove gelatin solution from Transwell chambers paying attention not to damage the membrane.
8. Add 600 µl of FN-free medium in each well of the 24-well plate, position the Transwell insert and seed 200 µl of cell suspension in the inner chamber. Incubate the cells for 24 hours at 37°C. Add also two Transwell inserts with FN-free EGM-2 medium without cells (*see* **Note 3**).
9. Put in charge the battery of Millicell ERS-2 overnight accordingly to manufacturer instructions.

3.4 Check integrity of endothelial cell monolayer

1. Prepare Millicell ERS-2 system for the measurement disconnecting it from the charger, since leaving the meter plugged in during use causes undesirable electrical noise, which results in unstable readings.
2. Test the meter functionality at the beginning of each series of measurements connecting the test electrode, turning the power on and setting the MODE switch to Ohms (Ω).

3. If the meter is not displaying 1000 Ω, adjust the R. Adj. screw until the system shows a reading of 1000 Ω.
4. 24 hours after seeding put the 24-well plate containing Transwell inserts and the charged Millicell ERS-2 meter under the laminar flow hood. Allow cells to come to RT.
5. Immerse the electrode tips in 70% ethanol solution for 15 minutes. Allow to air dry for 15 seconds under laminar flow hood and rinse it in sterile PBS.
6. Immerse the electrode shorter tip in the Transwell inner chamber and the longer tip in the outer well. Take care that the shorter tip does not contact cell monolayer on the membrane and the longer tip touches the bottom of the outer well. To ensure stable and reproducible measurements, make sure to hold the electrode steady and at a 90° angle to the plate bottom.
7. Record the measurement in cell culture insert without cells, to determine blank resistance, and across the other inserts.
8. To obtain AEC monolayer resistance, subtract the mean value of resistance read across the blank inserts from the resistance read across the cell culture inserts with cells. The unit area resistance is obtained by multiplying the monolayer resistance by the effective surface area of the filter membrane, in the case of 24 well the surface is 0.3 cm^2.
9. If the unit area resistance across the endothelial cell monolayer is between 15-20 $\Omega \cdot cm^2$ (*see* **Note 4**), remove the medium from the Transwell inner chamber and the external well and replace it with fresh FN-free EGM-2. Add 200 µl of FN-free EGM-2 inside the Transwell and 600 µl in the well.

3.5 Fibronectin secretion quantification

1. After 72 hours, remove the medium from the inner chamber of Transwell insert and from the external well with a pipette and collect it in microcentrifuge tubes.
2. Centrifuge the samples at 2.000 rpm for 5 minutes at 4°C and move the supernatant in a clean tube, discard the pellet.
3. Add 1 µg of Rabbit anti-Mouse antibody to each sample (spike normalization) and take 20 µl of medium from the sample collected in the inner chamber of Transwell insert and 60 µl from the

sample derived from external well, add SDS–Page Protein Sample Buffer, boil at 95°C for 5 minutes (*see* **Note 5**).

4. Load samples and molecular mass protein markers into 8% polyacrylamide gel wells for separation by electrophoresis and transfer proteins onto nitrocellulose membrane.
5. Saturate the membrane in TBS supplemented with 10% BSA, 0.1% Tween-20 for 1 hour at 45°C.
6. Cut the membrane at around 75 kDa in two parts, to separate high molecular weight proteins from low molecular weight ones.
7. Incubate the upper part of the membrane with anti-ED-A FN (IST9) 1:1000 in TBS, 0.1% Tween-20 for 1 hour at RT.
8. Wash the membrane three times in TBS, 0.1% Tween-20 for 10 minute each.
9. Incubate the lower part of the membrane with Goat anti-Rabbit and the upper with Goat anti-Mouse HRP conjugated secondary antibodies, both diluted 1:20000 in TBS, 0.1% Tween-20 for 45 minutes at RT.
10. Wash the two parts of the membrane three times in TBS, 0.1% Tween-20 for 10 minute each.
11. Incubate with Clarity western ECL substrate and record the chemiluminescence. The upper band around 250 kDa correspond to secreted ED-A-FN while the lowest around 50 kDa to the major chain of Rabbit anti-Mouse antibody used as loading control.
12. Calculate the optical density of each band corresponding to secreted FN and normalize for the corresponding Rabbit immunoglobulin major chain band. We usually display the results in terms of ratio between FN secreted apically or basolaterally in silenced vs. control cells (Fig. 1).

3.6. Immunofluorescence on Transwell membranes

1. Wash the Transwell insert twice with PBS.
2. Fix the cells on Transwell insert membrane in 4% paraformaldehyde in PBS for 15 minutes at RT.
3. Wash the Transwell insert three times in PBS.
4. Permeabilize cells on Transwell insert membrane in 0.1% Triton X-100 in PBS, keeping the 24-well plate on ice.

5. Wash the Transwell insert three times in PBS.
6. Carefully cut the Transwell membrane from the insert with a scalpel and place it onto a microscope slide.
7. Incubate it with 20 µl of anti-VE-cadherin and anti-ZO-1 polyclonal primary antibodies, both diluted 1:200 in primary blocking solution (see Materials n.28), for 1 hour at RT.
8. Gently remove the primary antibodies with a pipette and wash the membrane three times with PBS.
9. Incubate the membrane with 50 µl of Donkey anti-Rabbit Alexa Fluor 555 conjugated and Donkey anti-Rabbit Alexa Fluor 488 conjugated secondary antibodies both diluted 1:400 in secondary blocking solution (see Materials n.28), for 1 hour at RT.
10. Gently remove the secondary antibodies with a pipette and wash the membrane three times with PBS.
11. Incubate the membrane with 20 µl of DAPI diluted 1:10,000 or TO-PRO-3 diluted 1:500 in PBS, for 10 minutes at RT, to stain the nuclei.
12. Gently remove the solution with a pipette and wash the membrane three times in PBS.
13. Add on the membrane a drop of Fluoromount-G or Mowiol (approximately 20 µl) and place on the top a round coverslip.
14. Let the samples dry over night at RT covered by light.
15. Take confocal images (Fig. 2).

4 Notes

1. Recipe to prepare Mowiol medium: dissolve 6 g of glycerol (analytical grade) and 2.4 g of Mowiol powder (Calbiochem) in 6 ml of MilliQ water, add 12 ml of 0.2M Tris buffer, pH 8, and stir the solution for 4 hours. Let the solution rest for 2 hours. Incubate the Mowiol for 10 minutes at 50°C in a water bath and centrifuge the solution for 15 minutes at 5.000 g. Take the supernatant and freeze it at -20°C in aliquots.

2. The optimal silencing period may vary for different siRNA (24, 48, 72 or 96 hours after the second oligofection). Accordingly, it is necessary to seed a different number of cells in order to obtain a subconfluent monolayer at the end of the silencing.
3. Fill with PBS1X each empty well (if any) of the plate, to minimize the evaporation of culture medium.
4. The endothelial cell monocultures had comparatively low TER values (max 25 $\Omega \cdot cm^2$) than epithelial cells (around 500-600 $\Omega \cdot cm^2$).
5. If collected medium vary in volume among wells, because of differential evaporation, take equal sample in percentage of the supernatant from each one.

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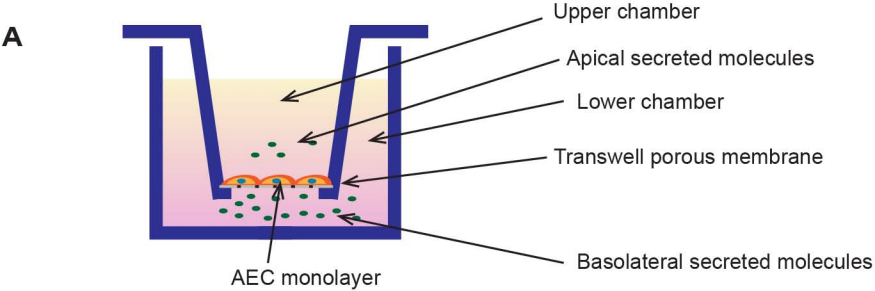
Figure legends

Fig. 1. Polarized FN secretion assay. **A**. Schematic representation of a well with a Transwell insert. **B**. Western blot analysis of the AD-A-FN secreted apically or basolaterally by confluent ECs seeded on Transwell inserts. An equal percentage of volumes of medium were collected after 72 hours of culture from inner and external chambers of different wells of siCTL or siPPFIA1 ECs. Rabbit IgG were added to each sample for loading control purposes. C. Quantification of the ratio between apical or basolateral amount of ED-A FN released by siCTL over siPPFIA1 ECs.

Fig. 2. Confocal microscopy characterization of AEC monolayer. AEC were seeded at the concentration of 20.000 (first lane), 50.000 (second lane) and 150.000 cells/well (third and fourth lanes) on the membrane of Transwell insert. 24 hours after seeding only the AECs seeded at the concentration of 150,000 cells/well formed a confluent monolayer (third lane) which is maintained intact after 96 hours (fourth lane). AECs were fixed on Transwell membrane and stained with VE-cadherin (red) and ZO-1 (green) Abs to visualize cell to cell contacts and TO-PRO3 (blue), to visualize nuclei.


A
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Lower chamber
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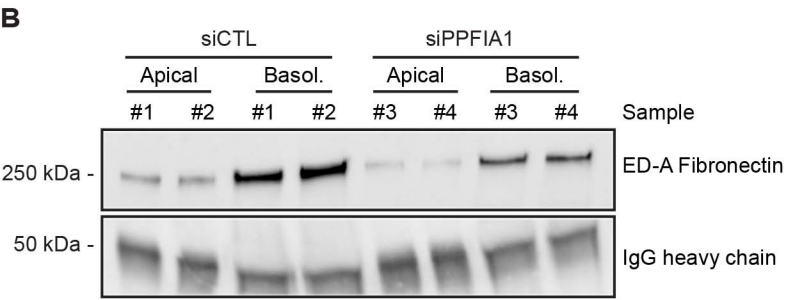
Basolateral secreted molecules
AEC monolayer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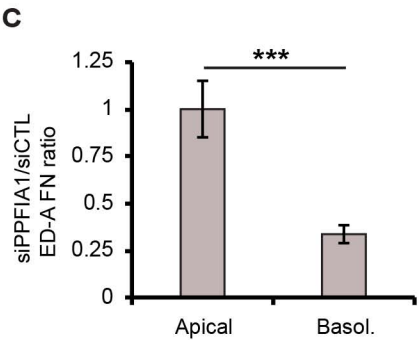
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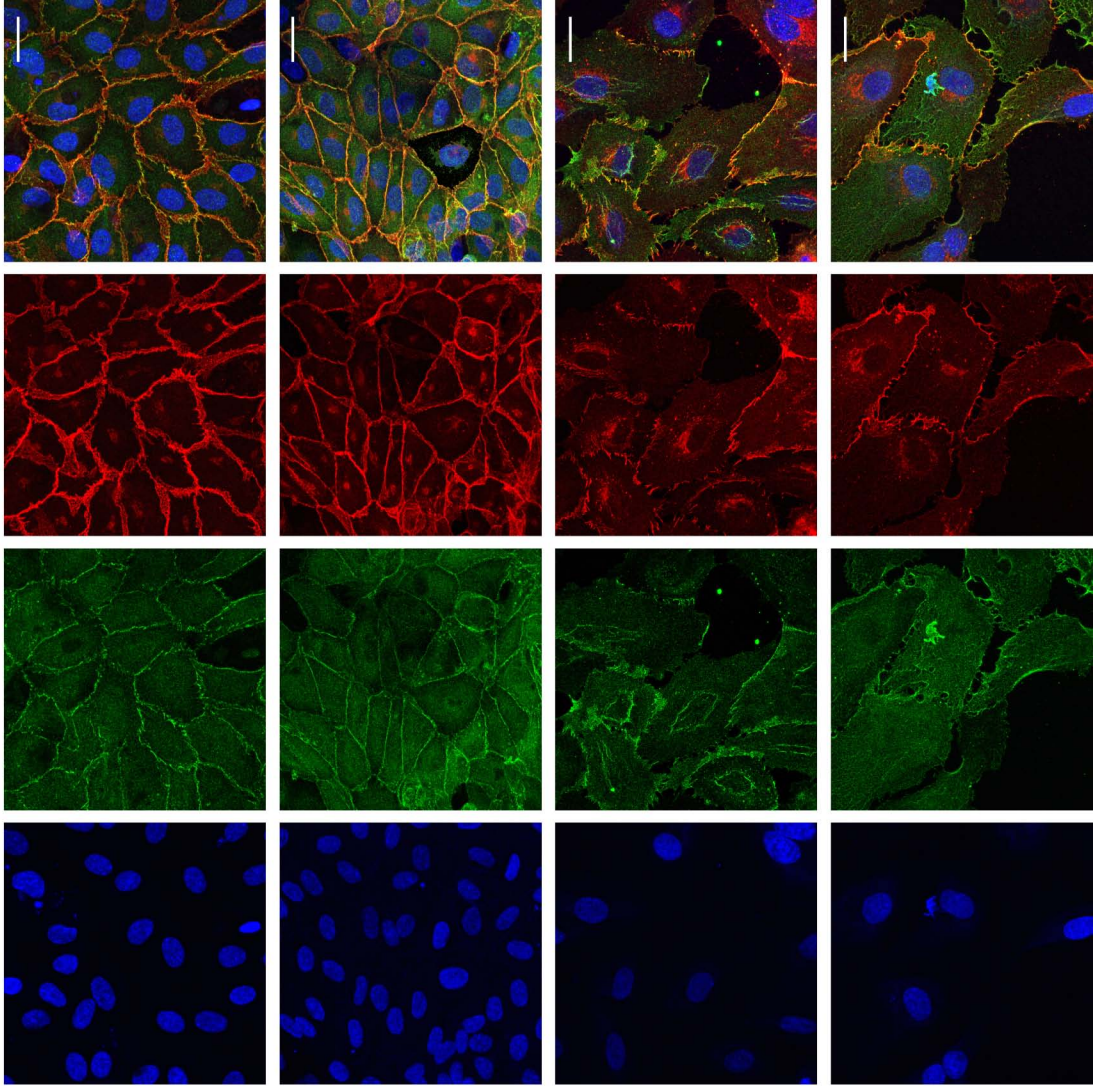
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VE-cadherin
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